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CONTRACT NUMBER DAMD17-94-C-4042

TITLE: Basic Research Effort Toward Development of a Vaccine Against Human Brucellosis

SUBTITLE: Identification of Protective Brucella Antigens and their Expression in Vaccinia Virus to Prevent Disease in Animals and Humans

PRINCIPAL INVESTIGATOR: Gerhardt G. Schurig, Ph.D.

CONTRACTING ORGANIZATION: Virginia Polytechnic Institute
Blacksburg, Virginia 24061-0249

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 May 96 - 30 Apr 97)

4. TITLE
Basic Research Effort Toward Development of a Vaccine Against Human Brucellosis
SUBTITLE: Identification of Protective Brucella Antigens and their Expression in Vaccinia Virus to Prevent Disease in Animals and Humans

5. FUNDING NUMBERS
DAMD17-94-C-4042

6. AUTHOR(S)
Schurig, Gerhardt G., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Virginia Polytechnic Institute
Blacksburg, Virginia 24061-0249

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This project identified several potentially protective *Brucella* antigens: YajC, GroEL, GroES, SOD, SecD, 18kDa, 16kDa TolB, L15, Pal. Some potential virulence factors have also been identified. Antigens have been purified in mg quantities as fusion proteins for further characterization. Selected antigens have also been cloned into Baculovirus for antigen characterization and immunization purposes. Vaccinia/*Brucella* recombinants with selected *Brucella* antigens have been produced and have been tested in mice. Mice are able to respond to these antigens immunologically but protection has not been achieved. New recombinants using more effective early/late synthetic promoters are being constructed to overcome this problem. Positive control vaccinia/listeriolysin recombinants are being constructed to assess vector efficacy. To assess the true protective abilities of the selected *Brucella* antigens, *Brucella abortus* vaccine strain RB51 disruption mutants have been constructed and DNA vaccines have been produced. These are being tested in mice for their ability to induce specific immune responses and protection against challenge with virulent *Brucella spp.*

14. SUBJECT TERMS	15. NUMBER OF PAGES
Brucella, Vaccinia Recombinants, Protection, Antigens, Immune Response, Vaccine.	22
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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9/28/97
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III. Table of Content.

V. INTRODUCTION.

a. Nature of the problem, background and previous work.

Brucellosis affects millions of people worldwide. Humans contract the disease either by consuming infected foods or by coming in contact with animals shedding the organism. The disease is characterized by an undulant fever, cold sweats and general malaise; any exercise will produce pronounced fatigue. If untreated the disease can last from a few weeks to several years. Serious complications leading to death can occur. Tetracycline is the treatment of choice for infected humans. In severe cases tetracycline treatment is supplemented with streptomycin or rifampicin. Brucellosis affects a variety of animals including swine, cattle, sheep, goats, dogs, and camels. Important species of *Brucella* are: *suis, abortus, ovis, melitensis, canis*, and *neotomae* each with certain predilection for a particular animal species.

Humans are susceptible to *B. melitensis, B. suis, B. abortus* and *B. canis* in decreasing order. The disease is endemic in many Middle East countries, Asia, Mexico, Central and parts of South America.

Antibiotic resistant mutants of *Brucella*, including tetracycline, rifampicin and streptomycin resistant ones, are not difficult to produce in the laboratory. Such mutants could be utilized in biological warfare resulting in high morbidity with no adequate treatment. Therefore, prevention of infection through vaccination is desirable.

Currently available vaccines can not be used in humans because of their side effects or lack of effectiveness. The current, most used vaccine for protecting cattle against *B. abortus* is strain 19 and for protecting goats against *B. melitensis* is strain Rev 1. Vaccination with these strains leads to seroconversion complicating diagnosis of the disease. Both of these vaccine strains are pathogenic for human beings. *B. abortus* strain RB5I was developed in our laboratory and has now replaced strain 19 (Federal Register 1996) as the vaccine of choice for bovine brucellosis. This strain is a stable, rough natural mutant devoid of 0-side chain. The strain is able to induce protective immunity in mice (1) and cattle (2,3) without the induction of antibodies to the 0-side chain (1,2). Transfer of T cells from strain RB5I immunized mice will transfer protective immunity against a virulent challenge (4), similar to the immunity transferred to mice by T cells derived from strain 19 immunized mice (5). This confirms previous indications that immunity to *B. abortus* infection requires a strong CMI component (4,5,6,7,8). The specific antigens which confer strong protective CMI have yet to be defined.

Macrophages are the principle cells of residence for *Brucella* in infected animals. Resistance usually depends on the correct interaction between T lymphocytes, specific for particular bacterial antigens, and the macrophage (9,10,11,12,13,14). Activation of macrophages by interferon-gamma (INF-γ) will lead to the destruction of intracellular *Brucella* (4). This further suggests that T cells, particularly those responsible for the production of INF-γ (T helper1-CD4+ response) are of major importance in anti-*Brucella* immunity (4). These observations do not eliminate a concomitant protective role for cytotoxic T cells (Tc, CD8+), since protective immunity can be demonstrated by passive transfer of either Th or Tc cells (8). It is possible that in Brucellosis the major role of the Tc cells in protection is production of INF-γ and not direct cytotoxicity. Considering these observations, it is most likely that *Brucella* proteins involved in protective immunity will preferentially stimulate INF-γ producing T cells which in turn activate macrophages enhancing their brucellacidal capabilities. Therefore, if *Brucella* proteins with such characteristics could be identified, they would likely induce a strong and protective CMI response. They could be used in cloning and expression systems that are able to present these antigens appropriately to the immune system.

b. Purpose of current work.

This project intends to identify *Brucella* antigens which are likely to stimulateTh1 responses (with production of INF-γ) with lymphocytes from vaccinated mice and therefore, are likely to have a role in the induction of protective immunity against brucellosis. Once such antigens have been identified, the genes encoding those antigens will be isolated, sequenced and their products will be characterized. Using these genes, recombinant vaccinia viruses will be constructed. These vaccinia recombinants will be used to immunize mice to determine whether they can induce a humoral and/or CMI response and protect mice against a challenge with virulent *B. abortus*.

c. Outline of approach.

The methodology used in this project and particularly during this reported period can be outlined as follows:

I. Identification of potentially protective *Brucella* antigens. For this purpose, two different strategies were followed.

i). Express *Brucella* antigens in genomic expression libraries and detect potentially protective antigens by either or both:

1. Reactivity of the recombinant clones with specific antibodies of the IgG2a subisotype found in immunized animals (IgG2a is considered

as a potential indicator of Th1 involvement) and

2. Ability of the protein extracts of the recombinant clones to stimulate the lymphocytes from animals immunized with the protective *B. abortus* strain RB5l vaccine. Lymphocytes are tested for their ability to proliferate and produce INF-γ upon exposure to such antigens. We also added cytotoxicity assays to detect induction of T cytotoxic (Tc) cells and flowcytometric analysis.

II). Subclone such genes identified by the above outlined procedures into vaccinia virus (Western Reserve and/or Wyeth strains). Test the recombinants for their ability to express the antigens *in vitro* and their ability to induce a protective immune response in mice. Analyze the character of the immune response induced by the recombinant vaccine and if protective, analyze in detail the immunological parameters associated with protection.

III) In order to have unequivocal results for in vitro CMI analysis, antigens have to be purified. Two main approaches are being used: overexpression of specific genes in *E.coli* as fusion proteins of either thioredoxin (pThio-His expression system, Invitrogen, Inc.) or maltose binding protein (pMal expression system, Novagen) and purification of the recombinant proteins on metal or amylose affinity chromatography. An additional approach is to produce the antigens of interest in bacculovirus.

VI. Body.

Detection of potentially protective antigens.

This project has detected various *Brucella* proteins which could be involved in the protective immune response. Genes of most of these proteins have now been sequenced and characterized. Some of these genes are being cloned into vaccinia and some genes have already been cloned into vaccinia for protection and immune response studies. These proteins were selected because they induced all or some of the following responses: T cell proliferation, production of INF-γ and production of IgG2 antibodies, in *Brucella* vaccinated mice. These antigens are: YajC, SecD, 18kDa, 14kDa, GroEl, GroES and Cu/Zn SOD. We have now established that the complete gene of the 14kDa protein actually codes for a 16 kDa protein. The 18kDa is an outer membrane lipoprotein.

Last year we described several recombinant clones in *E. coli* expressing

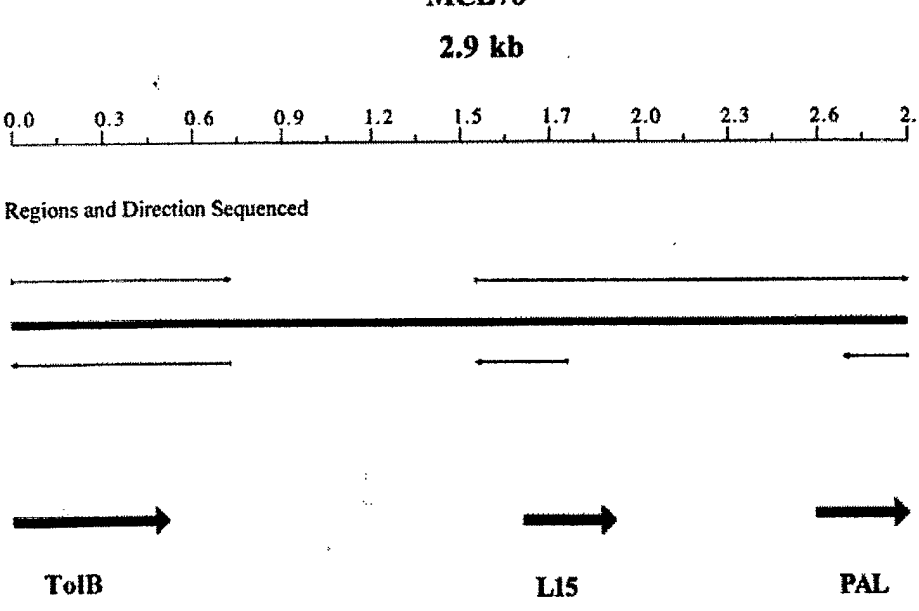
Brucella Immuno reactive proteins. These clones fell into 3 general groups based on their *Brucella* insert size when digested with the enzyme (ClaI) originally used for constructing the library:

group 1: Containing a 2.7 kb fragment
group 2: Containing a 2.9 kb fragment
group 3: Containing a 3.8 kb fragment consisting of a 1.1 and a 2.7 kb fragments

Southern blot analysis indicated that the 2.7 kb fragments from groups 1 and 3 clones were the same.

One of the clones from group 1 (clone MCB68) was selected for further study. Sequence analysis indicated that the insert was part of the SecD operon. Based on the sequence analysis, genes present in this clone were found to be of YajC and SecD proteins. There is 28 and 33% amino acid sequence similarity between *Brucella* and *E. coli* SecD and YajC proteins, respectively. In *E. coli* ,*secD* encodes for a 67 kDa SecD protein responsible for membrane transport; *yajC* encodes a hypothetical protein of 12 kDa of unknown function.

One of the clones from group 2 (clone MCB76) was also selected for further study since restriction mapping of the insert revealed a 2.9 kb insert that was different from other MCB clones. The proteins produced by MCB76 gave positive lymphocyte proliferation results, induced INF-γ production and IgG2a antibodies against them were detected in the ELISA. Sequencing of approximately 70% (both strands) of the MCB76 has been completed.



Sequence analysis of this region revealed that there are 3 possible open reading frames encoding polypeptides with significant homology to the genes encoding TolB (38% similarity to colicin transport), L15 (17% similarity; ribosomal protein on 50S subunit) and Pal (26% similarity; peptidoglycan associated lipoprotein). Only the L15 gene appears to be complete, whereas the 30-50 % of the other two genes are not on the insert (see figure above). From a protective antigen point of view, one or all 3 proteins might have been responsible for the observed, positive *in vitro* immune responses . However, *tolB* seems less of a vaccine candidate as its 5' end including the promoter region is absent and argues against any type of fortuitous fusion protein having been formed during subcloning. Thus we would not anticipate seeing any portion of the *tolB* gene expressed in *E. coli*. On the other hand, both the *L15* (intact) and the *pal* genes (50% including the 5' end) could be expressed from their own promoters.

It is anticipated that the rest of the insert (800 bp) will be sequenced within a month as all the deletion clones have been prepared. We will decide whether we should proceed with making one or more fusion proteins for purposes of testing them as protective antigens. One means to choose between L15 and Pal proteins is to assay the deletion clones (prepared for sequencing) for immuno-reactivity in one or more of the *in-vitro* immunological tests. Only the gene(s) that remain positive will be used for making gene fusions or vacinnia constructs. It is therefore possible that we may have additional proteins for further studies.

In order to detect further antigens possibly associated with protection but which may not be detectable by the above outlined screening methods (appearance of specific antibodies or detection of reactive T cells using proliferation or INFγ detection assays) we have also looked for potential virulence factors of *Brucella*. Immune responses to virulence factors do not have to be necessarily associated with protection but, often protective antigens are virulence factors. Therefore, isolation, identification and characterization of *Brucella* genes that may be responsible for the intracellular survival and or ability of the parasite to persist in animals appears to be pertinent to this project.

Brucella abortus chromosomal library in pBR322 (sal) and a smaller *Brucella melitensis* chromosomal library in pBlueScript (HindIII) in *E.coli* were tested for their ability to survive in mice for 48 hr after intraperitoneal (ip) injection. Approximately 10 to 12 *E. coli* clones were pooled and injected simultaneously into mice in order to accommodate the large number of clones being tested for increased virulence. The *E.coli* strains with just the vectors (pBR322 and pBlueScript) served as controls. It was determined that 10^8 colony forming units (c.f.u) of these control *E.coli* were cleared within 24 hours, when given ip.

Twelve pools of *E. coli* clones from the *B. abortus* library and four pools from the *B. melitensis* library survived for over 48 hours in the spleens of mice after ip inoculations. The vast majority of the pools were cleared from the mice within 48 hours. At this stage we are concentrating our efforts on 4 clones able to survive in mice for more than 48 hours and which were isolated from the *B. melitensis* library. The clones were selected by isolating individual colonies on plates and testing for the presence and characterization of their plasmids.

Table1. Preliminary Analysis of Sequencing data for the following four pBSMB clones

Clone # the clone	Insert Size in kb (Fig.1)	Completed Sequence (bp)	Homology
pBSMB31	~6-7	~1939	VACB Protein
pBSMB28F	~5-6	~712	ATP synthase
pBSMB16	~1.6	~1265	Transcriptional Regu
pBSMB12	~2.8	~800	unknown

From the above results, it can be concluded that three clones (pBSMB 31, pBSMB 28, and pBSMB 16) may be associated with intracellular survival or virulence in general. We will continue to study these clones and genes they contain to asses if they are involved in protection.

Purification of antigens.

As mentioned before, selection of antigens as candidates of protective antigens is based on the ability of T cells from *Brucella* immunized mice to react with the antigens *in vitro* in proliferation assays and induction of INF-γ. In order to insure that the selected antigens are inducing these responses, it is important to have these antigens in pure form which can be achieved by using recombinant protein expression systems. In order to purify the expressed recombinant proteins, a practical route is to express them as fusion proteins. For this purpose we are overexpressing the selected *Brucella* genes in *E.coli* as fusion proteins of either thioredoxin (pThio-His expression system) or maltose binding protein (pMal expression system) and purification of the recombinant proteins on metal or amylose affinity chromatography. This was done with commercial kits and modifications were introduced to optimize protein yield (into several mgs) and purity. Quantification of purified fusion proteins is carried out using BioRad microtiter protein assays and purity is assessed by Western blot analysis. We have now large quantities (in mgs range) of the following fusion proteins available for continuous testing:

GroES::Thio-his, YajC::MBP, Cu/Zn SOD::Thio-his, Lysteriolysin::Thio-his and GroEL::MBP. The GroEL::MBP is missing approximately 30 aminoacids at the C terminal end of the GroEL. Because of this problem we are also producing a GroEL::Thio-his. Future work will use this GroEl fusion protein instead of GroEL::MBP.

Purification of recombinant fusion proteins from *E. coli* even by affinity chromatography can not always guarantee 100% purity. This is a problem if impurities have biological effects on our *in vitro* immune response testing system. Also, the fusion partner may influence the *in vitro* response. In order to have an additional antigen purification method which would not utilize fusion proteins, we selected the expression of antigens in Baculovirus (BV). Also, *in vitro* recombinant BV production systems utilize components (insect cells lines, insect virus) which are unlikely to stimulate non-specific reactions in our *in vitro* testing systems.

The BAC-TO-BAC Baculovirus expression system of GibcoBRL (Grand Island, NY) was used to yield Baculovirus recombinants. It is based on site-specific transposition of an expression cassette into a BV shuttle vector (bacmid) propagated in *E. coli*. Genes to be expressed are inserted into the multiple cloning site of pFASTBAC1 downstream from the polyhedrin promoter.

B. abortus-pFASTBAC1 donor plasmids were generated for the 60 kDa molecular chaperon GroEL, the 10 kDa GroES and the 54 kDa heatshock protein HtrA. The identity of the cloned *B. abortus* genes were demonstrated by hybridization of digoxygenin-labeled DNAs to Southern blotted restriction enzyme digests of the pFASTBAC1 donor plasmids and recombinant bacmids.

Spodopternfrugiperda (Sf9) cells were transfected with the recombinant bacmid DNA using CELLFECTIN reagent. Recombinant virus was harvested at 72 hrs. To analyze the protein expression, large volume of cultures was infected with recombinant viruses and the cells were harvested at 48 hours post-infection. Samples of lysed and infected cells were analyzed on 10 % or 15 % SD S-PAGE gels followed by Western blotting. Western membranes were incubated with polyclonal goat anti-B. *abortus* , polyclonal goat anti-GroES (Goat 52), monoclonal mouse anti-GroEL, or monoclonal mouse anti-HtrA antibodies. Bound antibodies were detected with goat anti-mouse IgG or rabbit anti-goat IgG, conjugated to horseradish peroxidase, using 4-choloro- 1 -naphthol. Recombinant viruses were titrated using *Sf9* cells and the titers were calculated by the method of Reed and Muench and expressed in median infective dose per ml (TCID50/ml)). We have used the Sf9 cells infected with *Brucella* antigen expressing BV as antigens for *in vitro* lymphocyte transformation assay using lymphocytes from un-immunized normal mice and have determined that these preparations will not cause non-specific stimulations of lymphocytes. This opens the possibility that the *Brucella* expressing BV can be used as specific

antigens in our *in vitro* systems. Preliminary mouse immunization experiments with the recombinant BV indicate that these viruses can induce specific immune responses to the *Brucella* proteins.

Cloning into Vaccinia virus

In addition to the production of vaccinia recombinants expressing *Brucella* proteins reported previously we have constructed a GroEl/ vaccinia recombinant which expresses the whole GroEl protein using the Wyeth strain. We discovered that this recombinant would not replicate in mice therefore new constructs were made with the Western Reserve vaccinia using an early/late vaccinia promoter p7.5 (fig 1) during this report period.

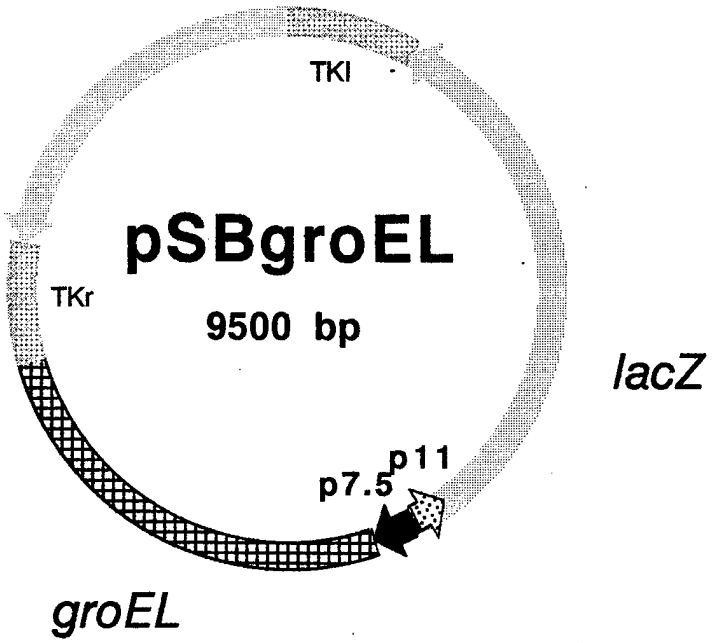


Fig 1. Diagram of recombinant plasmid pSBGroEL. A 1.7 kb fragment containing the *B. abortus groEL* gene (cross hatched line) was cloned into the shuttle vector pSC11 (light gray regions). The early/late vaccinia virus promoter p7.5 (solid arrow) regulates expression of the *groEL gene* and a late vaccinia virus promoter p11 (dotted arrow) regulates the expression of the *lacZ* gene. The vaccinia virus thymidine kinase sequences (TKl, TKr) flank the expression cassette controlled by the p7.5 and p11 promoters.

This recombinant, denominated WRSBGroEL, was tested for its ability to induce specific antibodies and selected cell mediated immune responses in mice as well as being able to protect these animals against challenge.

Construction of WRSBGroEL (similar strategies are being followed with some modification for the production of additional vaccinia/*Brucella* recombinants).

In order to prepare the vaccinia virus expression vector coexpressing *B. abortus* GroEL heat shock protein, plasmid pBA2168 was digested with restriction enzymes *EcoR* I, *Sal* I and *Dra* II . Overhanging ends were filled in and the resulting 1.7 kb fragment containing the *B. abortus gro*EL gene was purified from 1 % agarose gel , the fragment was ligated into the *Sma* I site of shuttle plasmid pSC11 to produce plasmid pSBGroEL. Human thymidine kinase deficient 143B cells (HuTK$^-$ cells) were grown to 80% confluency in Eagle's Minimum Essential Medium (EMEM) containing 5% fetal bovine serum (FBS) in 25 cm^2 flasks, infected with vaccinia virus strain Western Reserve (WR) (ATCC) at a multiplicity of infection (MOI) of 0.05 and incubated. One µg of pSBGroEL was dissolved together with lipofectin, incubated for 25 minutes at room temperature and the mixture (100µl) was added to 1 ml of EMEM and added to WR infected HuTK$^-$ cells at 80% confluency. After a 4+ cytopathic effect (CPE) had developed , the cells were ruptured by 3 cycles of freezing in liquid nitrogen and thawing at 37^0 C. The cell lysates, containing the putative recombinant virions, were serially diluted in 10-fold steps and subcultured onto a new monolayer of HuTK$^-$ cells in flat-bottom six-well plate with EMEM containing 25 µg of bromodeoxyuridine (BdUR) per ml for selection of recombinant virus. Following a 4+ CPE development, the medium was aspirated and the infected cells were overlaid with 1 ml of plaquing media (2x EMEM with 50µg BdUR) containing 0.6 mg/ml of Bluo-gal. Blue plaques, produced by replicating recombinant virions expressing the *lacZ* gene, were collected and used to enhance the virus content of the plaques by inoculating a confluent layer of HuTK$^-$ cells in either 25 cm^2 flasks or six well tissue culture plates. Replication of the recombinant virus was assessed by CPE and the presence of blue plaques in the cell monolayer. Recombinant virus was harvested, plaque purified and enhanced by infecting larger volumes of cell monolayers two more times to develop the recombinant virus WRSBGroEL. Same experiments were also carried out to generate the vaccinia virus recombinant WRSC11 (control) with shuttle vector pSC11.

Recombinant virus was tested for infectivity, purity and ability to induce GroEL antibodies in mice. 10^4 plaque forming units (pfu) of recombinant virus were injected into each of three BALB/C mice. One of the mice was killed six days later and the recombinant virus was isolated from ovaries and tested for CPE and β-galactosidase activity in HuTK$^-$ cells. Mouse was confirmed infected. The second mouse was killed on day thirteen post infection and the same procedures were followed in order to isolate and to test the infectivity of the recombinant virus in the cell culture. The third mouse was kept alive to obtain serum for serological tests. After isolating the virus from the second mouse, the

recombinant virus was plaque purified again and enhanced three more times in order to reach 10^7pfu/ml, the desired infective dose for mice vaccination. Recombinant expression of Brucella GroEL was confirmed by western blotting using anti- B. abortus strain RB51 goat serum (fig2).

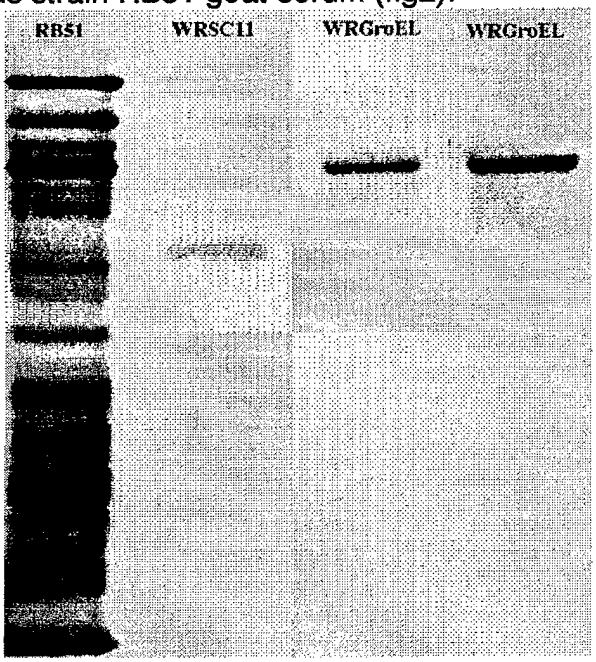


Fig 2. Western blot analysis of vaccinia virus recombinant WRGroEL (2 different stocks) and plasmid control (WRSC11) and *B. abortus* strain RB51 antigens with goat anti-RB51 serum

Immunization for protection studies were carried out as follows: Five groups of 8 female BALB/C mice each were used. The first and second groups were injected with 10^7pfu of WRSBGroEL intradermally (id) and intraperitoneally (ip) respectively. The third group was inoculated ip with 10^7pfu/ml of vaccinia virus/shuttle plasmid recombinant WRSC11. The fourth group received 2.6 x 10^8 colony forming units (cfu) of *B. abortus* rough strain RB51 (ip) as a positive control for protection and the fifth group received saline ip. Five mice out of each group were challenged with virulent *B. abortus* 8 weeks post immunization and killed 2 weeks later to asses the number of colony forming units (CFU) in the spleen. The remaining mice were used for serology and in vitro lymphocyte proliferation using various antigens including GroEL::MBP as the specific antigen. GroEL:MBP was missing the last 30 carboxyl end aminoacids of GroEL.

Results can be summarized as follows: Mice vaccinated with the WRSBGroEL developed IgG class antibodies specific for *Brucella* GroEL, lymphocyte proliferation assays results were not conclusive and protection was not observed. Further studies are underway to understand why protection was not

induced. The lack of *in vitro* lymphocyte stimulation may be related to the use of GroEL::MBP as the *in vitro* stimulating antigen, as mentioned above the GroEL was missing the last 30 aminoacids and it is possible that the stimulatory epitopes are concentrated in that area. The detailed work with this recombinant is available in a recent MS thesis (Assessment of the expression of Brucella abortus heat shock protein, GroEL, in vaccinia virus to induce protection against a Brucella challenge in BALB/c mice. Simge Bologlu, Master of Sciences, Virginia Tech, June 1997).

In general, we now believe that the nature of the promoter used (p7.5) does not lead to the appropriate *in vivo* expression levels of GroEL needed to induce an appropriate CMI response and therefore, no protection is observed. This possibility has to be explored before more complex issues like appropriateness of GroEL presentation when delivered by vaccinia are investigated. For this reason, new vaccinia recombinant constructs are being carried out using a synthetic early/late promoter.

As mentioned above, the lack of a strong specific CMI response in mice vaccinated with the recombinant vaccinia viruses could be due to the nature of the promoters present on the cloning vectors used in the preparation of the recombinant viruses. Recently, shuttle vectors (pMCO2 and pSC65) with a synthetic early/late promoter were developed. Glucoronidase is the marker enzyme in pMCO2 vector whereas in pSC65 it is β-galactosidase (fig 3). Expression of the cloned genes by the recombinant vaccinia viruses prepared with these new vectors is 1000 times over natural early or late vaccinia promoters.

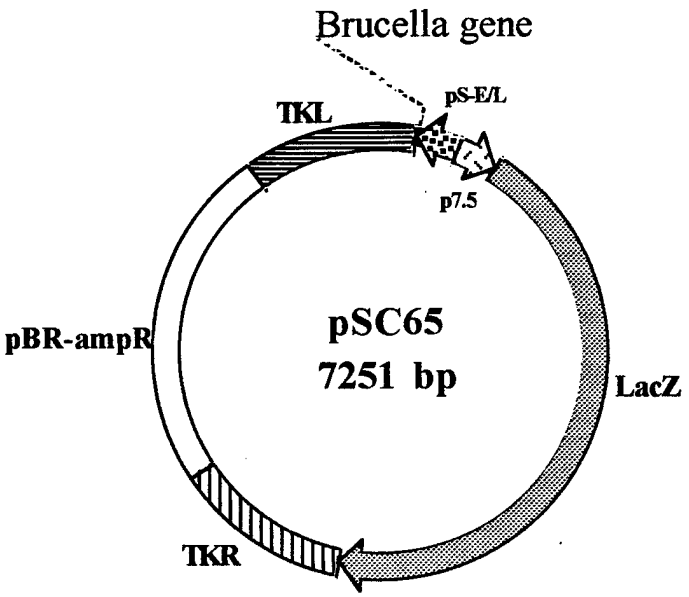


Fig. 3. Schematic representation of the vaccinia shuttle vector pSC65 where *Brucella* genes can be cloned under a synthetic early/late promoter.

Note: pMCO2 contains glucoronidase as the marker enzyme, whereas pSC65 contains β-galactosidase.

Higher levels of *Brucella* protein expression by the recombinant vaccinia viruses should enhance the specific and hopefully appropriate CMI responses in the vaccinated mice leading to a better chance of protection against a virulent *Brucella* strain challenge.

Based on this rationale, we cloned the 18kDa gene in shuttle vector pMCO2 and recombinant vaccinia virus was constructed using the WR strain (vWRMC18). Mice were vaccinated ip 10^7 pfu/mouse with vWRMC18 and bled at several time intervals post vaccination. Strong antibody responses were observed against the 18kDa antigen and the vaccinia virus antigens confirming replication of the virus as well as Brucella antigen recognition (fig. 4).

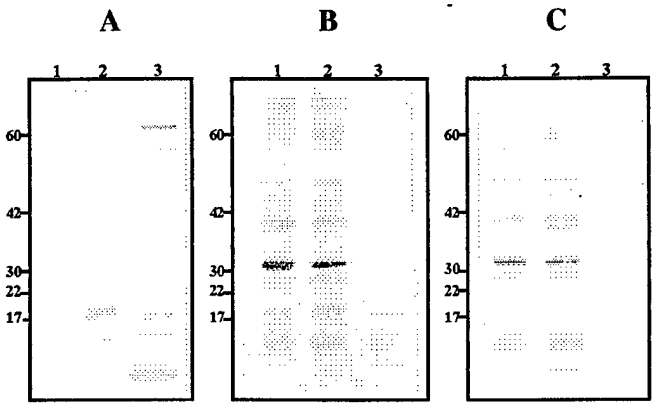


Fig. 4. Extracts from cells infected with the recombinant vaccinia virus containing the 18 kDa protein gene either in the wrong orientation (lane 1) or in the right orientation (lane 2), and *B. abortus* strain RB51 (lane 3) were reacted with sera from mice vaccinated with *B. abortus* strain RB51 (panel A), the recombinant vaccinia virus expressing the 18 kDa protein (panel B), and the recombinant vaccinia virus not expressing the 18 kDa protein (panel C).

Preliminary flowcytometric analysis of whole blood lymphocyte from vaccinated mice indicate that the CD4+ cells are responding to the specific 18kDa protein but not the CD8+ cells as indicated by the number of CD69 expressing cells while the total number of CD4+ and CD8+ remains constant (tables 2 and 3).

Lymphocytes obtained from the spleens of these mice proliferated *in vitro* upon stimulation with purified MBP-18 kDa fusion protein and produced IFN-γ into the culture supernatants. The *in vitro* CMI results combined with the strong antibody

responses to 18 kDa, indicate that the synthetic promoter is achieving better results than the promoters used before and encouraged the use of the promoter in future constructions.

Table 2. Percentages of CD69-expressing cells in CD4+ and CD8+ T cells, in peripheral blood, 4 weeks after vaccination. Cells were activated with 3.6 ug/mL 18 KDa protein for 8 hr.

	CD4	CD8
Vaccinia 18 KDa	3.2	0.5
Vaccinia RB51	1.4	0.4
	3.2	0.5
PMA+Iono m.	27	0.8

Table 3. Percentages of Total CD4+ and CD8+ T cells, in peripheral blood, 4 weeks after vaccination.

	Total CD4	Total CD8
Vaccinia 18 KDa	25	7.5
Vaccinia RB51	20.9	6.6
	23.5	4.5
PMA+Iono m.	ND	ND

Production of INFγ is considered to be one of several crucial immunological events necessary to obtain protective immunity against *Brucella*. However, when challenged with the virulent *B. abortus* strain 2308, these mice did not show any level of protection. Although the immunization with vaccinia/18kDa protein did induce an *in vitro* INFγ response to stimulation with 18kDa::MBP, one can not assume that protection must be observed. Other immunological events may have to occur simultaneously, like the production of specific cytotocic T cells, to obtain protective CMI. We have now standarized cytotoxity

assay in the laboratory and are testing for the presence of *Brucella* specific cytotoxic lymphocytes in recombinant vaccinia immunized mice. Establishment and standardization of this test using 51Cr and / or neutral red release from mouse macrophage cells lines (targets) took several months of work.

The lack of protection inspite of the development of strong humoral and cell mediated immune responses to the 18kDa protein could therefore be related to the following two reasons among others:
a) the 18 kDa antigen is not at all involved in the induction of protection against *Brucella* infection or
b) the recombinant vaccinia virus is unable to express *Brucella* antigens in a way leading to correct processing an induction of protective CMI.

To test the first possibility, we constructed a 18kDa-disruption mutant of vaccine strain RB51 by inserting a kanamycin cassette into the 5' end of the 18kDa gene. Mouse protection experiments performed with this mutant RB51 strain, showed similar level of protection as those obtained with the vaccine strain strongly suggesting that the 18 kDa antigen is not involved in the elicitation of a protective immune response. This is probably the most likely explanation for the protective failure of vWRMC18. The protection results obtained from this experiment emphasize the importance of using antigens of proven protective capabilities for the construction of the vaccinia recombinants. Therefore, we are now also constructing RB51 disruption mutants using those antigens selected for cloning into vaccinia to asses if they play a significant role in protection. Obviously, no disruption mutants can be obtained with genes which are essential to *Brucella* survival (*groEL, groES*).

Along the same line, we are additionally testing the protective ability of the selected *Brucella* antigens by vaccinating mice with plasmid DNA (pCDNA, Invitrogen) containing the specific *Brucella* genes under an eukaryotic promoter (cytomagaloviral promoter) and other essential transcriptional signal sequences. Currently we have immunized mice with DNA vaccines of GroEL and SOD using a variety of injection routes. Initial analysis indicated that all the immunized mice developed a strong antibody response against the specific proteins. CMI assay are being planed and the mice in this pilot study will be challenged with virulent *Brucella* to assess protection. We are initially targeting the GroEL, GroES, and SOD proteins since homologues of these proteins from other intracellular pathogens have been shown to be protective if a suitable antigen delivery system is used (15, 16, 17). It has been clearly demonstrated that delivery of appropriate antigens through DNA vaccination results in immune responses necessary for protection against several viral as well as bacterial pathogens (18,19).

To address possibility b), the recombinant vaccinia virus is unable to express *Brucella* antigens in a way leading to correct processing of an induction of

protective CMI, we will be testing the effectiveness of our vaccinia delivery system by constructing a recombinant virus expressing the listeriolysin protein of *L. monocytogenes*. Listeriolysin is a well demonstrated protective antigen in murine listerial infections (20). The gene for listeriolysin was PCR amplified from the genomic DNA of *L. monocytogenes* strain EGD. Primers for the amplification were designed based on the published sequence information (21). The part of the gene coding for the signal sequence was not included for the amplification. The amplified fragment was cloned in vaccinia shuttle vector pSC65 and recombinant virus was prepared using WR strain. Upon an initial screening, several recombinant viral plaques expressing the marker enzyme β-galactosidase were obtained. Clear expression of listeriolysin by these recombinant viruses is yet to be demonstrated. The use of recombinant vaccinia virus expressing listeriolysin in mice should demonstrate protection against challenge with *L. monocytogenes* and serve as a positive control for our vaccinia delivery system. Failure to protect mice against *L. monocytogenes* challenge by this recombinant would indicate that our vaccinia system is not an appropriate delivery system for protective *Brucella* antigens and that further research has to be carried out on the expression system itself before a successful vaccine can be engineered.

Publications generated during this report period:

Thesis: Assessment of the expression of *Brucella abortus* heat shock protein, GroEL, in vaccinia virus to induce protection against a Brucella challenge in BALB/c mice. Simge Bologlu, Master of Sciences, Virginia Tech, June 1997.

Vemulapalli R, Duncan AJ, Boyle SM, Toth TE, Sriranganathan N, Schurig GG. Isolation and expressional cloning of *secD* and *yajC* genes of *Brucella abortus*. Abstract# B-106, 97th General meeting, American Society for Microbiology, Miami Beach May 4-8, 1997.

Cravero S, Vemulapalli R, Toth TE, Calvert CL, Boyle SM, Sriranganathan N, Rosetti O, Schurig GG. Specific immune responses of BALB/c mice inoculated with recombinant vaccinia virus expressing *B. abortus* 18 kDa outer membrane protein. Abstract# E-73, 97th General meeting, American Society for Microbiology, Miami Beach May 4-8, 1997.

Vemulapalli R, Lopez-Santiago R, Moreno-Lafont M, Toth TE, Sriranganathan N, Boyle SM, Schurig GG. Characterization of immune responses of mice vaccinated with vaccinia virus expressing 18 kDa antigen of Brucella abortus. Brucellosis conference 1997.

VII. Conclusions.

During this period we have been able to discover several additional *Brucella* antigens potentially involved in a protective immune response as indicated by

in vitro correlates of CMI. Some of these "antigens" may be virulence factors. The antigens were purified in mg quantities as fusion proteins which allow us to characterize and understand the immune response to these putative protective antigens. An alternate method of producing specific *Brucella* antigens using recombinant Baculovirus, also appears useful for such studies. Studies with pure antigens are important to insure that we are dealing with antigens which will induce appropriate immune responses.

Studies with some of our vaccinia/*Brucella* recombinants expressing these putative protective antigens constructed in our laboratory clearly indicate that they can express *Brucella* antigens, that mice immunized with the recombinants are able to make an immune response against these antigens but, that protection has not been induced. One possible reason for this protective failure is that the promoters selected to achieve expression are not strong enough. New constructs are being made using early/late synthetic promoters to overcome this problem. It is also possible that the vaccinia system as applied in this project, may not be appropriate to induce the right, protective cell mediated responses. This area is being explored by constructing vaccinia recombinants expressing listeriolysin. These recombinants should protect against challenge with *L. monocytogenes*. Depending on the results obtained, we may have to further alter the vaccinia expression system to achieve protection or, we may have to further concentrate on the protective nature of the selected *Brucella* antigens. In order to clearly conclude that the selected *Brucella* antigens do play a role in protection, selected disruption mutants of protective *B. abortus* strain RB51 are being constructed and DNA vaccines are being prepared. We were successful in producing both and they are being tested in mice for their ability to induce CMI and protection against challenge. Loss of protection using specific RB51 disruption mutants or protection afforded with DNA vaccines would strongly suggest that our selected antigens are of protective nature. Initial experiments with our DNA vaccines indicate that immunized animals do produce humoral and/ or CMI responses to the specific antigens tested.

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